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BIOACTIVITY OF THE RED ALGAE *Asparagopsis taxiformis* COLLECTED FROM THE SOUTHWESTERN COAST OF INDIA

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ABSTRACT

Among the diverse variety of red algae, *Asparagopsis taxiformis* constitutes one of the abundant biomass in the Kollam coast (Southwest coast of India). Therefore, in the present study, *A. taxiformis* was collected, extracted and fractionated using column chromatography. The individual fractions were evaluated *in vitro* for their antifouling, anticyanobacterial, piscicidal and crustaceans toxicity assays. The fraction eluted with 2:8, petroleum ether and ethyl acetate exhibited strong and broad spectrum of bioactivity. In antifouling assay against *Limnea truncatula*, the active algal fraction produced 80% of foot repellency at 150 mg/L whereas in anticyanobacterial assay, the active fraction inhibited 100% growth of *Trichodesmium* sp. at 320 mg/L. The algal fraction showed higher piscicidal effect at the level of 60 mg/L. The crustacean toxicity of the active fraction was also evaluated to find compounds without toxicity in non target organisms, *Penaeus monodon* and *Macrobrachium rosenbergii*. It was found that column fraction showed less toxicity against the non target organisms. The chemical constituents of the active fraction were identified by means of chromatographic systems such as TLC, reverse phase HPLC and GC-MS. The overall activity profile envisages that the active column fraction of *A. taxiformis* might contain synergistic bioactive metabolites that could be utilized for the control of fouling organisms, algal bloom and herbivorous/predaceous fishes in aquaculture ponds.

RESUMO

Entre as diversas variedades de algas vermelhas, *Asparogopsis taxiformis* constitui uma das que apresentam alta biomassa na costa de Kollam (Sudoeste da Índia). No presente estudo *A. taxiformis* foi coletada, seca e reduzida a pó, após o que foi realizada sua extração e feito o fracionamento usando-se cromatografia por coluna. As frações individuais foram avaliadas *in vitro* em ensaios para testar sua capacidade anti-incrustante, anticianobacteria e toxicidade para peixes e crustáceos. A fração extraída com eter de petróleo e etil acetato (2:8) apresentou o espectro de bioatividade mais forte e amplo. No ensaio anti-incrustação efetuado com com o molusco pulmonado *Limnea truncatula*, a fração algal ativa produziu 80% de repelência do pé em 150 mg/l, enquanto que no ensaio anticianobacteria a fração ativa inibiu 100% do crescimento de *Trichodesmium* sp., em 320 mg/l. A fração algal mostrou o efeito mais intenso contra peixes no nível de 60 mg/l. Em relação aos crustáceos, a toxicidade da fração ativa foi avaliada também visando encontrar compostos não tóxicos para organismos não alvo, tais como *Penaeus monodon* e *Macrobrachium rosembergii*. Foi visto que a fração ativa da coluna mostrou menor toxicidade para estas espécies. Os componentes químicos da fração ativa foram identificados por meio dos sistemas cromatográficos, tais como TLC, fase reversa do HPLC e GC-MS. O perfil geral de atividade aponta que a fração ativa da coluna para *A. taxiformis* pode conter metabolitos bioativos sinérgicos que podem ser utilizados para o controle de organismos incrustantes, explosão algal e peixes herbiboros/predadores em tanques de aquicultura.

Descriptors**:** *Asparagopsis taxiformis*, Lipophilic compound, Antifouling activity, Anticyanobacterial activity, Piscicidal activity.
Descritores: *Asparogopsis taxiformis*, Fração algal, Atividade anti-incrustante, Atividade anticianobactéria, Atividade piscicida, Toxicidade para crustáceos.

INTRODUCTION

Biofouling, algal blooming and herbivory pose a substantial technical, economic and ecological threat to marine habitats, shipping and mariculture industries worldwide. Biofouling alone causes an annual global loss of more than $6.5 million in the maritime domain (BHADURY; WRIGHT, 2004). Similarly, algal blooms may have many harmful effects including the development of high biomass and scums, acute toxicity to fish and shellfish, suffocation of fish and crustaceans from mucus production and gill interference (GLIBERT, 2007). It is estimated that the annual economic loss from serious water bloom events in the United States alone is usually over several billion U.S. dollars **(**GEOHAB, 2001). Algal bloom caused by *Trichodesmium* sp. has been a regular phenomenon on Indian coasts, leading to the death of fish and having other environmental and economic impacts. The herbivorous/predaceous fishes in culture pond are distributing worldwide, causing serious threat for culturing shrimps/prawns, especially in nursery ponds. The predatory fish species prey on cultured stock whereas the competitors compete for food, space and oxygen. These species are extremely harmful when present in sufficient numbers. Moreover, some of the herbivorous/predaceous fishes are vectors for many shrimp parasites including microsporidian (FLEGEL et al., 1992).

Currently, many synthetic chemicals are widely deployed for the control of biofouling, algal blooming and herbivory/predatory, however only a few of them are of practicable use due to their high cost, secondary pollution, or impracticability and persistence in the environment. Considering the emerging issues pertaining to the use of synthetic chemicals, suitable environmentally benign substitutes are an urgent need. The use of marine natural products that are capable of deterring these noxious organisms may provide a solution to these environmental issues. Data pertaining to the antagonistic relationship of marine secondary metabolites to fouling and grazing organisms were noted as earlier as the 1980s (GERWICK; FENICAL, 1980; TARGETT et al., 1983).

Seaweeds are the most primitive group of the vegetation which came into existence in the pre-Cambrian era and contribute 90% of the existing species of marine plant, being crucial primary producers in the oceanic food web. They are able to biosynthesize secondary metabolites that can mediate a broad range of intra and inter specific ecological interactions between marine organisms, including chemical defenses against herbivores (HAY; STEINBERG, 1992; PAUL, 1992) and anti-fouling interactions (NYS et al., 1991, 1998). Although thousands of bioactive compounds have been discovered, the need for novel bioactive compounds is still urgent as regards synthetic chemicals for managing biofouling, algal blooming and grazing.

The extensive southwestern coast of India (the Kollam coast) has been recognized as the exclusive habitat for diverse seaweeds but assessments of their biological activity have been rare and restricted to very few publications (MANILAL et al., 2009a). Among the diverse varieties of seaweeds, the red algae, *Asparagopsis taxiformis* constitutes the most abundant biomass on the coast. *A. taxiformis* releases a variety of bioactive compounds during its growth cycle, several chemical compounds, possessing various biologically relevant activities, having been isolated from it (MCCONNELL; FENICAL, 1977; WOOLARD et al., 1979; LATURNUS et al., 1996; EL-BAROTY et al., 2007; GENOVESE, 2009). However, very little is known about the functions of these metabolites in their natural environment. Moreover, in our previous work, out of fifteen red algae screened for antimicrobial activity, *A. taxiformis* from the southwestern coast of India was found to be the most active (MANILAL et al., 2009a). The present study was, therefore, designed to investigate the biological activity of *A. taxiformis* and the potential development of novel, environment-friendly formulations useful in the control of algal bloom, biofouling, herbivorous/predaceous fishes of aquaculture ponds.

MATERIAL AND METHODS

Collection and Extraction of Seaweed Bioactives

Asparagopsis taxiformis was collected from the intertidal and subtidal habitat of the Kollam (08º 54' N and 76º38'E) area (Fig. 1) located on the southwestern coast of India. The collection was performed during the period from December 2007 to April 2008 when red algal diversity remains dominant. Live and healthy plants were harvested manually and washed thoroughly in running water to remove epiphytes, animal castings, sand, and calcareous and other adhering detritus matters. Cleaned plant materials were shade dried under an air jet to prevent photolysis and thermal degradation. The completely dried material was weighed and ground coarsely in a mechanical grinder.

The extract of *A. taxiformis* was prepared by standardized procedure as described by Manilal et al. (2009a). Briefly, 2000.0 g of the dried seaweed powder was weighed and submerged in a flask containing 5000 ml of methanol (purity grade 99 %) and placed at 35ºC in a shaker at 120 rpm for two weeks. After two weeks, the crude extract was filtered using a paper filter fitted with a Buchner funnel using suction pressure, followed by centrifugation (Eppendorf) at 6000 *x* g for 5 min at 20ºC. The

supernatant was collected in a round-bottomed flask and the remaining solvent was concentrated up to 5-10 ml in a rotary vacuum evaporator (Yamato). The residue collected was evaporated to complete dryness in a vacuum desiccator and stored in the refrigerator.

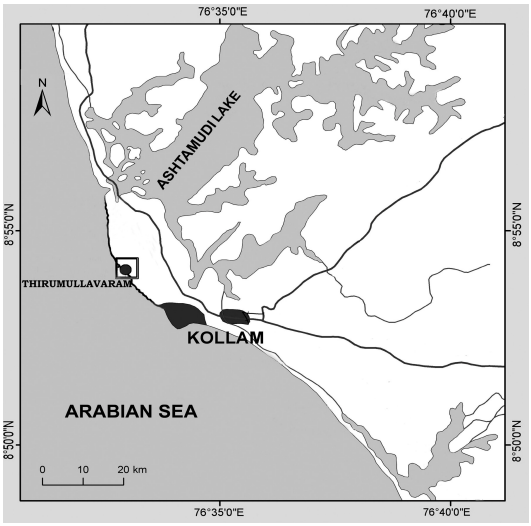

Fig. 1. Map showing the study area, southwest coast of India.

Phytochemical Analysis of *A. taxiformis*

The methanolic extracts of *A. taxiformis* (200.0 gm) was applied in a silica gel (60-120 mesh) column developed with petroleum ether and eluted with petroleum ether and ethyl acetate (9:1 to 1:9 and 100% ethyl acetate) followed by ethyl acetate and methanol (9:1 to 1:9 and 100% methanol) yielded seven fractions. Individual fractions were collected and tested for bioassay (data not shown). The fraction that was eluted using petroleum ether and ethyl acetate (2: 8) retained the highest activity was further purified by preparative TLC using silica gel G as the stationary phase with 1% methanol in dichloromethane as the mobile phase. After the development of the chromatogram, the resolved spots were analyzed by spraying with 50% sulphuric acid for detecting the lipophilic compounds (MANILAL et al., 2009a). The TLC resolved spots were recovered by scrapping off the adsorbent at the appropriate place on the developed plate and eluted with methanol and centrifuged at 10000 *x g* for 5 min. The supernatant with activity was subjected to HPLC (Shimadzu chromatographic system, Kyoto, Japan) and GC-MS analysis (Hewlett Packard). The compound identification was achieved on the basis of comparison of retention indices, as well as by computerized matching of the acquired mass spectra with those stored in the NIST Version 2.0 (2005).

Bioassay screening of *A. taxiformis*

Antifouling Assay

The effect of the algal fraction on foot repellent activity against the common fouler *Limnea truncatula* was tested as an index of antifouling activity. Snails of 3.0±0.3 mm in shell length were collected during low tide from the rocky substratum of the Kollam coast and acclimatized in a 1000 L FRP tank filled with seawater and maintained at optimum temperature (28-30ºC), salinity (35‰) and aeration, for three days. Each of the test samples of different concentrations (100, 150, 200 m/L) was evenly distributed in one half of the petridish (80 mm diam.) to obtain a uniform film of extracts and the other half was treated with methanol and dried overnight at room temperature. The assay plates were filled with 4 ml of filtered seawater and kept on an illuminated transparent glass surface for the visual observation of mobility and foot adherence of the treated snails. The mobility of the snails involves recognition of suitable surfaces and the production of biological adhesives substances that ensure attachment. Ten snails were carefully placed in the centre of each assay plate. Each experiment was carried out in triplicate. Assay plates were kept at room temperature (28±2°C) for 2 h. The number of snails on both treated and untreated halves was recorded after two hours.

Piscicidal Assay

Fingerlings of the common culturable fish, *Oreochromis mossambicus* (Peters) were collected from prawn ponds on the southwestern coast of India. Fingerlings (1.5-2.0 cm) of *O. mossambicus* were laboratory acclimatized and used for evaluating the ichthyotoxic potential. Five fingerlings were introduced into each of the experimental and control glass bowls containing 1000 ml of seawater in which a chosen concentration (20, 40, 60, 80 mg/L) of the extract had been dissolved. Immediate behavioral changes and mortality were observed continuously for six hours and at 1 h intervals for the next 12 h. After 24 h of exposure, the numbers of dead and live fish were counted. The behavioral changes of treated fishes were observed and recorded.

Anticyanobacterial Assay

As a cyanobacterial sample, *Trichodesmium* sp. was collected from shrimp ponds of Kollam (Munroe Island) and isolated and maintained in a marine broth (Himedia). The cyanobacterial cells were diluted to $1x10^5$ cells and inoculated in a marine broth

containing a chosen concentration of the algal fraction in 250 ml Erlenmeyer flasks and kept at 23±2ºC with shaking at 60 rpm, 12:12 h photoperiod and 2000 lux light intensity, for seven days. On the seventh day, the cell density of cyanobacteria was calculated using a haemocytometer under a zoom stereo microscope. Untreated medium and medium with 1% ethanol served as the positive and negative controls.

Crustacean Toxicity Assay (Non-target Organisms)

A bioassay for the determination of seaweed toxicity was conducted using the culturable shrimp *Penaeus monodon* (Fabricius). A batch of apparently healthy shrimps of unknown sex was maintained under optimum environmental condition in a 1000 L FRP tank provided with constant aeration. Ten juvenile shrimps were introduced per experimental tank (10 L glass aquaria) containing 5000 ml of reconditioned seawater in which a chosen concentration (0.5, 1, 2, 3, 4 g/L) of the extract had been dissolved. Aeration and temperature were kept constant and physical changes of the treated shrimp were monitored and recorded at two-hour intervals. The same assay method was applied to the freshwater prawn *Macrobrachium rosenbergii* (de Man) using filtered rain-fed pond water.

Results and Discussion

Study Area

The study area, the southwestern coast of India, is an ideal habitat for diverse endemic marine flora and fauna. A recent survey undertaken after the 2004 tsunami by Mantri (2006) reported 31 genera represented by a total of over 45 species of seaweed at the collection sites on the Kollam coast. Among the species reported, the red algae represent a considerable part of the algal biomass present in this region throughout the year. Although much research has been carried out into their taxonomy and morphology, very few studies have, to date, examined their bioactive potentials (MANILAL et al., 2009 a, b).

Antifouling Activity

The results of the foot repellence assay demonstrated that the *A. taxiformis* fraction tested displayed effective repellence to *L. truncatula* (Table 1). The column fraction of *A. taxiformis* repelled the snails strongly even at very low concentration, confirming the test as a useful indicator of general toxicity against fouling organisms. During the two hours of the test period, *L. truncatula* showed unequal contact with both the treated and untreated regions of the petridish. As the attachment process involves recognition of a suitable surface to spread their foot for adherences, the percentage of repellency was estimated based on the spreading of the foot. At a dose level of 150 mg/L the algal fraction exhibited 80% repellency against *L. truncatula.* In higher concentrations, the animal fails to spread the foot on the surface, whereas in lower concentrations even though they spread and attach their foot but immediately move to the control region of the petridish. There was a significant increase in the number of *L. truncatula* in contact with the solvent control region of the petridish as compared to the number of snails on the corresponding part treated with the algal fraction. The present result therefore suggests that *A. taxiformis* had repellent properties against rock fouling organisms. Similar results were reported for the red algae, *H. musiformis* by Selvin and Lipton, (2004) against *Patella vulgata*. Similarly, a study by Cho et al. (2001) demonstrated that the methanolic extracts of two seaweeds *Ishige sinicola* and *I. okamurae* can cause 100% foot repellant activity at 40 µg concentrations. Many marine biogenic compounds have been isolated and shown to exercise antifouling activity against marine foulers (RITTSCHOF, 2001), but their ecological role and antifouling properties have not yet been fully studied (NYS; STEINBERG, 2002). Katsuoka et al. (1990) ascertained that two compounds isolated from the seaweed *Costaria costata*, galactosyl and sulfo-quinovosyl-diacylglycerols, exercised activity against mussels. Therefore, based on its repellent activity, the alga *A. taxiformis* could be utilized as a renewable natural resource for the development of an antifouling agent. It is of great importance that the bioactivity of *A. taxiformis* against a large diversity of foulers should be studied, as agents with a wide spectrum of activity are sought for antifouling purposes.

Table 1. Antifouling activity of *A. taxiformis* against *L. truncatula.*

Concentration of seaweed (mg/L)	Untreated	Treated
100	78.4±3.6	15.65±2.8
150	89.7±4.4	11.33±3.2
200	100±0.0	0.0±0.0

Mean ± SD $n = 6$ experiments

Piscicidal Activity

Secondary metabolites from a wide variety of benthic organisms have been reported to deter feeding by natural predators (PAUL, 1992; PAWLIK, 1993; HAY, 1996). In the present study, the *in*

vitro ichthyotoxic potential was estimated as the ability of an algal fraction to kill fish at a particular concentration. The results meant that the algal active fraction produced its toxic effect at a lower concentration. At a dose level of 60 mg/L, *A. taxiformis* produced 100% mortality in tilapia after 2 h. In the highest dose levels (60, 80 mg/L), the treated fishes showed initial behavioral changes including reduced swimming endurance, hyperventilation and rapid erratic movement, began after 30 min of exposure. During the later phase (1.5-4 h), in highest dose levels, the fishes swam up with sudden surface gasping and show paralysis-like signs. Shortly thereafter, the fishes turn upside down and finally inclined to the base followed by death.The mode of action of the algal fraction may be due to its inhibition of the central and peripheral nervous system of the fish. The results suggest that piscicidal activity may be due to the presence of lipophilic algal secondary metabolites. Lara-Isassi et al. (2000) reported that, among the 58 species of Mexican seaweeds studied, genera of Rhodophytes showed potent ichthyotoxic activity. Similarly, Selvin and Lipton (2004) reported that the crude extract of *Hypnea musiformis* produced 20% mortality in tilapia at 2 mg/ml. The crude extract of *Dictyota dichotoma* from the southwestern coast of India has been reported to exercise antifeedant activity at 50 µg/ml against the fish *Danio aequipinnatus* (MANILAL et al., 2009). The indiscriminate usage of synthetic piscicides possess many detrimental effects such as bioaccumulation (BOYD; TUCKER, 1998; ARASTA et al., 1996), long residual life, discoloration and off flavor to the flesh (CULLEN; CONNELL, 1992). During the last few decades increasing attention has been paid to extractives from natural sources particularly botanicals, which offer an alternate strategy to the prevalent use of synthetic piscicides. The biodegradable phytoproducts such as tea cake seed and derris powder have been proposed as natural piscicides substance (SHIGUENO, 1975) in shrimp/prawn ponds but are high cost and less available in market (TIWARI; SINGH, 2005).

Based on the present findings, it may be inferred that the red alga *A. taxiformis* can reduce the grazing pressure of herbivorous fishes and its piscicidal defense mechanism makes it a potential candidate for the development of eco-friendly piscicides.

Anticyanobacterial Activity

In the present study, the active column fraction of red alga *A. taxiformis* totally inhibited the growth of *Trichodesmium* sp., a nuisance cyanobacterium in shrimp ponds, at a dose level of 320 mg/L (Table 2). In a comparison with our study, El-Baroty et al. (2007) reported antialgal activity of *A. taxiformis* against the cyanobacteria, *Microcystis aeruginosa.* It has been reported that chemicals produced by some algalspecies can inhibit the growth both of algae and invertebrates as well as slow or stop larval development of bivalves (NELSON et al., 2003). Many studies have been undertaken to evaluate the antialgal property of seaweeds (TANAKA; ASAKAVA, 1988; LUSTIGMAM; BROWN, 1991; HELLIO et al., 2002). Cho et al. (1999) reported that a methanol extract of *Ishige sinicola* at 200µg/ml greatly inhibited growth of *Isochrysis galbana*. The poly-unsaturated fatty acids from *Ulva* sp. showed potent algicidal activity (ALAMSJAH et al., 2005). Similarly, Jeong et al. (2000) demonstrated that at a concentration of 50µg/ml the methanolic extract of *Corallina pilulifera* can inhibit the growth of the toxic microalga, *Cochlodinium polykrikoides*. Being less toxic to the crustaceans, the result obtained in the present study suggests that the extract of *A. taxiformis* has the potential to eradicate noxious algal bloom in coastal aquaculture ponds.

Table 2. Anticyanobacterial activity of *A. taxiformis* against *Trichodesmium* sp.

A. taxiformis	Density of cells in various concentration (mg/L)		
	100	200	300
	2.1×10^2 cells /ml	1.6×10^2 cells /ml	1×10^2 cells /ml
Cell morphology	-	Shrinkage of cell	Disturbed cell wall

Mean ± SD *n* = 6 experiments

Crustacean Toxicity

One of the major findings of the present study relates to crustacean toxicity. The activity of the *A. taxiformis* column fraction was mild and showed less toxicity against the non-targeted organisms such as *Penaeus monodon* and *Macrobrachium rosenbergii*. In the toxicity assay, *P. monodon* and *M. rosenbergii* were found to be more resistant to the algal column fraction and their LD_{50} values were, respectively, 1.8 g/L and 2.5 g/L after 24 h. We selected both species as non-target organisms in the present study, because both of them are economically valuable species of crustaceans culturable in aquaculture systems. The literature makes it evident that the effect of algal extracts on crustacean species has scarcely yet been tested. The greater tolerance/resistance of crustaceans may be due to their exoskeleton structure. Wisepongpand and Kuniyosh (2003) reported that at a dose level of 16µg/ml a phloroglucinol compound

isolated from the alga *Zonari desingiana* resulted in the mortality of the shrimp *M. lanchesteri* after 2 hours of treatment.

Chemical Nature of Active Fraction

Fractionation and Purification of *Asparagopsis taxiformis* extract

The crude extract of *A. taxiformis* was fractionated through column chromatography. The active fraction was purified using preparative TLC to obtain a single spot with an *Rf* value of 0.487. The active TLC resolved spot was again purified with reverse phase HPLC at 254 nm with methanol at a flow rate of 1 ml/min; head pressure at 25 kgf/cm^2. The whole setup was maintained at room temperature (25 °C) and three peaks with retention time (min) of 3.307, 3.863 and 4.730, respectively, were attained at 254 nm (Fig. 2). The resultant major peak was subjected to GC-MS analysis.

The gas chromatogram of the *A. taxiformis* is characterized by an intense peak at 34.89 min retention time, the major peak in the gas chromatogram, which has been assigned to 4,5-Dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (mol. wt 167). The other peaks in the gas chromatogram, of moderate and minor intensity, correspond to chlorobenzene (mol. wt 112); 14-methyl-pentadecanoic acid methyl ester (mol. wt 270); octadec-9-enoic acid 2,3-dihydroxy-propyl ester (mol. wt 265); 9-octadecanoic acid, methyl ester (mol.wt 255); octadecanoic acid methyl ester (mol. wt 209). The mass spectra of the compound confirmed with those available in the NIST library. In terms of GC identified chemical components, our results varied with the previous studies on *A. taxiformis* (Delile) Trevisan by El-Baroty et al. (2007). It could be attributed due to the different geographical region, the developmental stages and seasonal variations.

It was found that 4, 5-Dimethyl-1H-pyrrole-2-carboxylic acid ethyl ester (56.012%) was the major chemical component followed by pentadecanoic acid and octadecanoic acid which might be involved in synergistic bioactivity. Evidence supporting the bioactivity of fatty acids has already been demonstrated in certain microorganisms and fouling organisms (RUSSEL, 1991). Earlier studies have reported that many lower and higher fatty acids from seaweeds possess antimicrobial properties (KATAYAMA, 1960). Lipophilic extracts from seaweeds have been investigated as a source of substances with pharmacological properties (CACCAMESE et al., 1985; ROBLES-CENTENO et al., 1996; ARUN KUMAR et al., 2001; LIMA-FILHO et al., 2002; MANILAL et al., 2009b).

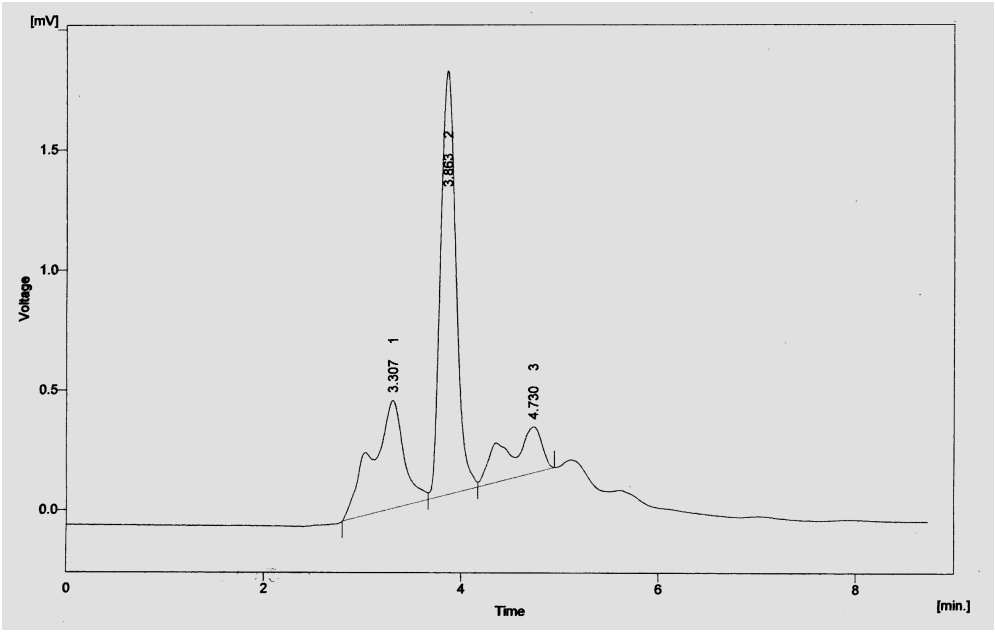

Fig. 2. HPLC Chromatogram of TLC fraction of *A. taxiformis.*

CONCLUSION

The results of the present study reveal that the marine red alga *A. taxiformis* is a potential source for a natural agrochemical development program as an alternative to the synthetic agrochemicals that are currently being used in fouler and weed control programs. However, nowadays no seaweed-based agrochemicals are in use for fouler and weed control. Such research work should focus on the Indian coast, where abundant unexplored algal resources exist. Therefore their mode of action and complete bioefficacy evaluation in field condition is further warranted.

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